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Sustained NFAT Signaling Promotes a Th1-Like Pattern of Gene Expression in Primary Murine CD4⁺ T Cells

Cynthia M. Porter² and Neil A. Clipstone³

T cell activation is known to be critically regulated by the extent and duration of TCR-induced signaling pathways. The NFAT family of transcription factors is believed to play an important role in coupling these quantitative differences in TCR-induced signaling events into changes in gene expression. In this study we have specifically investigated the effects of sustained NFAT signaling on T cell activation by introducing a constitutively active mutant version of NFATc1 (caNFATc1) into primary murine CD4⁺ T cells and examining its effects on gene expression. We now report that ectopic expression of caNFATc1 partially mimics TCR signaling, resulting in enhanced expression of CD25 and CD40 ligand and down-regulation of CD62L. More importantly, we find that expression of caNFATc1 in T cells maintained under either nonpolarizing or Th1-skewing conditions leads to a marked selective increase in the number of cells expressing the prototypical Th1 cytokine, IFN-γ. Furthermore, when expressed in Th2-skewed cells, caNFATc1 appears to attenuate Th2 differentiation by decreasing production of IL-4 and promoting the expression of IFN-γ. Finally, we find that caNFATc1 enhances expression of functional P-selectin glycoprotein ligand-1, up-regulates Fas ligand expression, and increases susceptibility to activation-induced cell death, cellular traits that are preferentially associated with Th1 effector cells. Taken together, these results suggest that sustained NFAT signaling, mediated by ectopic expression of caNFATc1, acts to promote a Th1-like pattern of gene expression and thereby serves to highlight the important relationship between the degree of NFAT signaling and the qualitative pattern of gene expression induced during T cell activation. The Journal of Immunology, 2002, 168: 4936–4945.

Antigenic stimulation of naive CD4⁺ T cells initiates a signal transduction cascade that ultimately results in the differentiation of distinct effector T cell populations capable of secreting discrete sets of cytokines that are in turn responsible for orchestrating an effective immune response (1). Th1 cells produce primarily IL-2 and IFN-γ and are involved in the regulation of cell-mediated immune responses, whereas Th2 cells primarily produce IL-4 and IL-5 and regulate humoral immunity (1). This process of Ag-induced T cell activation and differentiation proceeds via a complex genetic program of transcriptional events involving the regulated expression of numerous genes, including those encoding cytokines, cytokine receptors, and other immunoregulatory molecules (2, 3). Analysis of the cis-acting regulatory elements of many of these genes, including IL-2, IFN-γ, IL-3, IL-4, IL-5, IL-8, IL-13, TNF-α, GM-CSF, CD25, CD40 ligand (CD40L), and Fas ligand (FasL), has revealed the presence of numerous functional binding sites for the NFAT family of transcription factors (3, 4). Because this family of transcription factors is known to be highly inducible in response to TCR stimulation (3–5), NFAT family members are believed to play a pivotal role in the T cell activation-induced transcriptional response during Th cell differentiation.

NFAT proteins are regulated primarily at the level of their subcellular localization by a TCR-dependent signaling pathway involving the calcium/calmodulin-dependent phosphatase, calcineurin (3–5). NFAT family members are normally located in the cytoplasm of resting cells in a hyperphosphorylated latent form, but, following TCR stimulation and the ensuing increase in the intracellular calcium concentration ([Ca²⁺]), they are dephosphorylated by activated calcineurin, triggering their rapid nuclear import and causing an increase in their intrinsic DNA binding activity (3–5). This calcineurin-mediated activation pathway is strongly opposed by a number of specific protein kinases, which act to directly rephosphorylate NFAT proteins, resulting in a decrease in their intrinsic DNA binding activity and their rapid nuclear export, thereby attenuating NFAT-dependent transcription (6–11, 64). As a result of the opposing influences of calcineurin and the regulatory NFAT kinases, NFAT activity is readily reversible, highly dynamic, and extremely sensitive to changes in [Ca²⁺] (6, 7, 12). This aspect of NFAT regulation is likely to be of particular significance, because the extent and duration of NFAT activation have recently been shown to be important parameters that can significantly affect the qualitative pattern of gene transcription induced during T cell activation (13).

The calcium-regulated NFAT family of transcription factors is comprised of four known members: NFATc1 (NFATc/NFAT2), NFATc2 (NFATp/NFAT1), NFATc3 (NFATx/NFAT4), and NFATc4 (NFAT3) (14–18). To gain insights into the in vivo functional roles of the NFAT family, several groups have generated mice deficient in one or more of these NFAT genes. While these studies have revealed prominent functions for NFATc1 in cardiac...
valve morphogenesis (19, 20) and for NFATc2 in the regulation of chondrogenesis (21), the precise functions of NFAT family members in the immune system are less clear. Due to embryonic lethality of the NFATc1 knockout, the role of this protein in the immune system has been analyzed by the generation of chimeric mice using the RAG-1−/− blastocyst complementation system (22, 23). NFATc1−/− chimeric mice exhibit a decrease in thymocyte repopulation and impaired peripheral T cell proliferation. In addition, both splenocytes and lymph node cells from these mice have been shown to produce diminished levels of IL-4, but essentially normal levels of IL-2 and IFN-γ, in response to anti-CD3 mAb stimulation. These latter results suggest that NFATc1 is specifically involved in the regulation of Th2 cytokines. Mice deficient in NFATc2 develop age-related splenomegaly and accumulation of T cells with an activated phenotype (24, 25). NFATc2-deficient T cells exhibit a normal primary proliferative response but a greatly enhanced secondary response (24, 25). The absence of NFATc2 does not appear to have a significant effect on the production of either IL-2 or IFN-γ, although the production of IL-4 is markedly enhanced in secondary immune responses (25, 26). NFATc3 is primarily expressed in thymocytes and, correspondingly, NFATc3-deficient mice exhibit impaired thymocyte maturation (27). NFATc3-deficient peripheral T cells exhibit an activated phenotype but produce normal levels of activation-induced cytokines (27).

Overall, the mild immunological phenotypes of the individual NFAT knockout mice suggest a considerable degree of redundancy within the NFAT family. This notion is supported by the much more severe phenotypes of the NFAT double knockout mice. Thus, NFATc2/NFATc3−/− mice exhibit a severe lymphoproliferative disorder, and T cells from these mice are hyperactive and produce markedly enhanced levels of Th2 cytokines following primary stimulation (28). The enhanced cytokine production and hyperactive state of T cells from NFATc2/NFATc3−/−, and NFATc2/NFATc3−/− mice has led to the surprising notion that NFATc2 and NFATc3 might play inhibitory roles in the regulation of the immune response. Most interestingly, NFATc1/NFATc2 doubly deficient T cells were found to be profoundly impaired in their production of a large panel of cytokines including IL-2, IL-4, IFN-γ, IL-10, GM-CSF, and TNF-α (29), indicating that the activities of both NFATc1 and NFATc2 are essential, but largely redundant, with respect to activation-induced cytokine gene expression. Based upon these observations, while it is clear that NFAT proteins play a crucial role in the regulated transcription of T cell effector cytokines, there does not appear to be a simple causal relationship between an individual NFAT family member and the expression of a specific cytokine gene(s). Accordingly, the precise role of each individual NFAT family member and the contribution of spatiotemporally distinct patterns of NFAT signaling to the differential expression of cytokine genes during T cell differentiation remain to be fully established.

In this study, we have used retroviral-mediated gene transfer to introduce a constitutively active mutant form of NFATc1 (caNFATc1) into primary murine CD4+ T cells to examine the effects of sustained NFAT activity on the regulation of CD4+ T cell gene expression. Our findings indicate that sustained NFAT signaling caused by the ectopic expression of NFATc1 enhances a number of cellular traits that are preferentially associated with Th1 effector cells, including a selective increase in the number of cells expressing the prototypical Th1 cytokine IFN-γ (1), increased expression of functional P-selectin glycoprotein ligand-1 (PSGL-1) (30), enhanced FasL expression, and increased susceptibility to activation-induced cell death (AICD) (31–33). We also find that ectopic expression of caNFATc1 in developing Th2 cells decreases expression of IL-4 and promotes the expression of IFN-γ.

Collectively, these results suggest that sustained NFAT signaling, mediated by ectopic expression of caNFATc1, acts to promote a Th1-like pattern of gene expression in primary murine CD4+ T cells.

Materials and Methods

Mice

Six-week-old female C57BL/6J and BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and DO11.10 TCR-transgenic × Rag-1−/− BALB/c mice were obtained from Dr. T. Barrett (Northwestern University Medical School, Chicago, IL). The mice were maintained in specific pathogen-free facilities at Northwestern University Medical School in accordance with Northwestern University animal care guidelines.

Cell culture

Phoenix-Eco packaging cells and primary T cells were maintained at 37°C in 7.5% CO2 in DMEM (Life Technologies, Rockville, MD) supplemented with 10% (v/v) FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, 1% (v/v) nonessential amino acids, 2 mM L-glutamine, 10 mM HEPES buffer, and 50 μM 2-ME. Recombinant murine IL-2, IL-4, and IFN-γ cytokines were purchased from PeproTech (Rocky Hill, NJ). Anti-CD3 mAb (145-2C11), anti-CD28 mAb (clone 37-51), anti-mouse IL-12 mAb (C17.8), and anti-mouse IFN-γ mAb (XMG1.2) were all purchased from BD Pharmingen (San Diego, CA), and anti-mouse IL-4 mAb was purified from hydridoma clone 11B11 tissie culture supernatants.

Retroviral plasmid constructs

The retroviral expression vector pMSCV-GFP (34) was supplied by Dr. L. Van Parijs (California Institute of Technology, Pasadena, CA). To generate pMSCV-caNFATc1-GFP, an N-terminal hemagglutinin-tagged, C-terminal FLAG-tagged constitutively active version of NFATc1 containing serine to alanine substitutions in the conserved serine-rich domain and all three serine-proline repeats (NFATc1-mSRD-mSp3x, see Ref. 11) was inserted into pMSCV-GFP downstream of the murine stem cell virus (MSCV) long terminal repeat (LTR). The caNFATc1 cDNA used in this study is derived from the 716-aa human NFATc isoform originally isolated by Northrop et al. (15). The pMSCV-H-2K4 retroviral expression vector was created by replacing green fluorescent protein (GFP) in the pMSCV-GFP retroviral expression vector with PCR-amplified truncated murine H-2K4 cDNA from the pMACs Kk.II plasmid (Miltenyi Biotech, Auburn, CA). pMSCV-caNFATc1-H-2K4 was generated by introducing the constitutively active form of NFATc1 into pMSCV-H-2K4.

Purification and activation of primary murine CD4+ T cells

Splenocytes harvested from 6- to 10-wk-old mice and CD4+ T cells were purified to >97% purity using MACSelect CD4+ MicroBeads (Miltenyi Biotec), exactly according to the manufacturer’s instructions. Purified CD4+ T cells were activated in vitro in 24-well plates (2 × 106 cells/well) coated with anti-CD3 mAb (5 μg/ml) and anti-CD28 mAb (5 μg/ml) in medium supplemented with 10 ng/ml murine (m)IL-2. For Th1-polarizing conditions 5 ng/ml mIL-12 and 10 μg/ml anti-IL-4 mAb were added to the cultures, whereas 50 ng/ml mIL-4, 5 μg/ml anti-IL-12 mAb, and 5 μg/ml anti-IFN-γ mAb were added for Th2-polarizing conditions. Cells were removed from stimulation on the third day and expanded in medium containing IL-2 for a total of 7 days. Where indicated, cells were maintained in Th1- or Th2-polarizing conditions for the entire 7-day culture period.

Retroviral production and infections

The Phoenix-Eco ecotropic packaging cell line provided by Dr. G. Nolan (Stanford University, Palo Alto, CA) (35) was transfected with the appropriate retroviral construct using Lipofectamine PLUS (Life Technologies). Viral supernatants were collected at 48 h and stored at −80°C. Activated CD4+ T cells were spin-infected at 24 and 48 h postactivation by centrifugation at 2000 rpm for 1.5 h at room temperature with 1.5 ml viral supernatant containing 6 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO) and 10 μg/ml mIL-2. Where indicated, cells were infected in the presence of the appropriate Th1/Th2-skewing conditions. The viral supernatant was removed after the spin infection and replaced with medium containing 10 μg/ml mIL-2 and the appropriate cytokines and Abs as required.

Flow cytometric analysis

On day 7 after infection, cells were incubated with either medium alone or medium containing 12.5 ng/ml PMA plus 1 μM ionomycin as indicated.
After 8 h of incubation, the cells were stained with either fluorochrome-conjugated specific mAbs or appropriate isotype control Abs and analyzed by flow cytometry. Anti-CD25 mAb (7D4), anti-CD154 mAb (MR1), anti-CD62L mAb (MEL-14), anti-CD45RB mAb (16A), anti-FasL, and anti-H-2Kb mAb (36-7-5) together with appropriate isotype control Abs, were all purchased from BD Pharmingen, while anti-mouse H-2Kb Ab (H100-27.R55) was purchased from Miltenyi Biotec. P-selectin-Ig fusion protein and control CD45-Ig fusion protein were provided by Dr. G. Kan- sans (Northwestern University, Chicago, IL) and Cy5-conjugated anti-human IgM was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Samples were analyzed using a FACS Calibur flow cytometer and CellQuest software (BD Biosciences, Palo Alto, CA).

Intracellular cytokine assays and ELISA

CD4+ T cells were plated at 3 x 10^5 cells/well in a 24-well plate and restimulated with medium alone, 12.5 ng/ml PMA (Calbiochem, La Jolla, CA), 12.5 ng/ml PMA plus 1 μM ionomycin (Calbiochem), or 12.5 ng/ml PMA plus 1 μM ionomycin plus 10 μg/ml FK506 as indicated for 6 h at 37°C, with the addition of 15 μg/ml brefeldin A (Sigma-Aldrich) for the last 4 h. After fixation in 2% paraformaldehyde, cells were permeabilized by incubating twice in permeabilization buffer (PBS plus 0.5% BSA, 1 mM sodium azide, and 0.5% saponin) for 15 min at room temperature. The relevant anti-cytokine or isotype control Abs were added at 0.5 μg/ml in 1 x 10^6 cells in permeabilization buffer and incubated for 30 min at room temperature. Cells were then washed twice in permeabilization buffer and twice in wash buffer (PBS plus 0.5% BSA and 1 mM sodium azide), and then analyzed by flow cytometry. PE-conjugated anti-IL-4 (BDV4-1D11), allopurinol-conjugated anti-IFN-γ (XMG12.1), PE-conjugated anti-IL-2 (JE6-S5H4), along with the isotype control PE-conjugated rat IgG (A95-1), and allopurinol-conjugated rat IgG (R3-34), were all purchased from BD Pharmingen.

For ELISA, cells transduced with either the MSCV-H-2Kb or MSCV-caNFATc1-H-2Kb virus were purified using MACSelect K MicroBeads (Miltenyi Biotec), washed, and restimulated (1 x 10^5 cells/ml) with 12.5 ng/ml PMA plus 1 μM ionomycin in a 24-well plate. After 24 h the presence of IFN-γ and IL-2 in the culture supernatants was determined using IFN-γ (KM-INF2) and IL-2 (KM-IL2) ELISA reagents from Endogen (Cambridge, MA). Supernatants were serially diluted and the concentration of cytokine was determined in relation to a reference standard using protocols provided by the manufacturer.

Analysis of AICD

Cells transduced with either the MSCV-H-2Kb or MSCV-caNFATc1-H-2Kb viruses were collected on day 7 after infection and H-2Kb-positive cells were purified using MACSelect K MicroBeads (Miltenyi Biotec). Cells were plated at 3 x 10^6 cells/ml in a 24-well plate and incubated with medium alone or medium containing 12.5 ng/ml PMA and ionomycin for 24 or 48 h, at which point TUNEL assays were performed using a TdT-mediated dUTP nick-end labeling (TUNEL) assay (Roche, Indianapolis, IN), exactly according to the manufacturer’s protocol.

Results

Generation of a recombinant retrovirus expressing caNFATc1

To investigate the functional effects of sustained NFAT signaling on T cell gene expression, we have taken advantage of a calcineurin-independent, constitutively active mutant version of NFATc1 (caNFATc1; NFATc-mSRD-mSpX3 in Ref. 11) that is known to be constitutively localized to the nucleus and capable of binding DNA with high affinity (11, 36). A retroviral vector encoding caNFATc1 was generated by introducing the caNFATc1 mutant downstream of the MSCV LTR and upstream of an internal ribosomal entry sequence-enhanced GFP cassette, allowing for bicistronic expression of both caNFATc1 and enhanced GFP.

FIGURE 1. Schematic representation of the retroviral vectors. The caNFATc1 DNA is located downstream of the MSCV LTR and upstream of the internal ribosomal entry sequence-enhanced GFP cassette, allowing for bicistronic expression of both caNFATc1 and enhanced GFP.

Expression of caNFATc1 in CD4+ T cells affects the expression of CD25, CD40L, and CD62L

To assess the initial effects of caNFATc1 on CD4+ T cells, we first analyzed the expression of several activation markers whose expression levels are known to change following T cell activation. As shown in Fig. 2A, CD25 is expressed at low levels on cells infected with the control virus and is up-regulated following restimulation with PMA and ionomycin. In contrast, cells transduced with the caNFATc1 virus and left unstimulated exhibited a high level of CD25 expression that was comparable to that observed in stimulated control cells (Fig. 2A). This result indicates that ectopic expression of caNFATc1 is sufficient to drive high-level expression of CD25 in the absence of other overt signals and is likely to be mediated via the direct action of caNFATc1 on the transcription of the CD25 gene, as functional NFAT binding sites have been identified in the CD25 promoter (37). In addition to CD25, caNFATc1 also enhanced the expression of CD40L, although in this case the level of CD40L expression was further increased following stimulation with PMA and ionomycin, indicating that, while caNFATc1 can partially induce the up-regulation of CD40L, other additional signaling pathways are necessary for maximal expression. The enhancing effect of caNFATc1 on the expression of CD40L is consistent with the presence of multiple NFAT binding sites within the CD40L promoter region (38).

CD62L (L-selectin) expression is often used to distinguish between naive and memory cells, as it is expressed at high levels on naive cells and is rapidly down-regulated from the cell surface in response to Ag stimulation (39). However, it is probably better considered a marker of recent Ag exposure, as it can be re-expressed on activated T cells as they enter a stage of poststimulation quiescence (40). As shown in Fig. 2C, CD62L is expressed on a substantial number of nonstimulated control virus-transduced cells and is down-regulated following stimulation with PMA and ionomycin. By comparison, in the absence of stimulation, a significant number of cells transduced with the caNFATc1 virus appear to have lost expression of CD62L (Fig. 2C). Hence it appears that caNFATc1 is able to partially mimic T cell activation signals resulting in the down-regulation of CD62L, although the underlying mechanism responsible for this effect is not known. Taken together, these data indicate that the caNFATc1 mutant construct is transcriptionally active and capable of mimicking T cell activation signals to affect the expression levels of endogenous T cell activation marker genes.

Ectopic expression of caNFATc1 synergizes with PMA to induce expression of IL-2 in CD4+ T cells

Because NFAT was originally implicated in the transcriptional regulation of the IL-2 gene (3–5), we next examined the effects of caNFATc1 on IL-2 gene expression. In control virus-transduced cells, expression of IL-2 was undetectable in either nonstimulated or PMA-treated cells but was expressed at high levels following stimulation with both PMA and ionomycin, an effect that was
blocked by the addition of FK506 (Fig. 3). In contrast, PMA treatment alone was sufficient to induce the expression of IL-2 in caNFATc1-expressing cells, albeit in only a subset of the population (19%). As expected, the majority of these cells produced IL-2 following stimulation with both ionomycin and PMA. Notably, expression of caNFATc1 was able to render cells partially resistant to the inhibitory effects of FK506. These results indicate that by bypassing the requirement for calcineurin-mediated activation caNFATc1 can synergize with PMA to increase the expression of the endogenous IL-2 gene, although it is apparent that optimal cellular expression of IL-2 requires additional FK506-sensitive, calcium-dependent signaling events.

caNFATc1 selectively increases the number of CD4\(^{+}\) T cells producing IFN-γ

Having demonstrated an effect of caNFATc1 on both T cell activation markers and IL-2 production, we next wanted to test the effects of caNFATc1 on production of cytokines associated with Th cell subsets, namely IL-4 and IFN-γ. For these experiments CD4\(^{+}\) T cells purified from C57BL/6 mice were transduced with either the control or caNFATc1 retrovirus and expanded under neutral nonskewing cytokine conditions, so as not to favor differentiation toward either the Th1 or Th2 phenotype. As shown in Fig. 4A, following restimulation with ionomycin and PMA, T cells transduced with the control virus were found to predominantly produce IL-4 (39% of cells), with relatively few cells producing IFN-γ (10% of cells). Similar results were obtained when nontransduced cells were analyzed (data not shown). However, a striking difference in cytokine profile was observed when T cells expressing caNFATc1 were analyzed. In this case, a marked increase in the percentage of cells producing IFN-γ was detected (60 vs 10%; Fig. 4A). In fact, over the course of multiple experiments we observed that ectopic expression of caNFATc1 resulted in an average 4.8-fold increase (n = 8) in the percentage of cells expressing IFN-γ. Similar results to those described above were obtained when CD4\(^{+}\) T cells from BALB/c mice were used (Fig. 4B), indicating that the selective effect of caNFATc1 on production of IFN-γ is not dependent on genetic background. In contrast to its marked effect on the production of IFN-γ, expression of caNFATc1 appeared to have little effect on IL-4 production under these conditions, even though the IL-4 promoter is known to contain several functional NFAT binding sites (41, 42). However, it is interesting to note that, although the total percentage of IL-4 producers in cells expressing caNFATc1 changed very little, the percentage of cells expressing both IFN-γ and IL-4 was greatly increased, with a corresponding reduction in the number of cells producing only IL-4 (Fig. 4, A and B).

We were initially surprised by the large number of IL-4-expressing cells generated under neutral nonskewing conditions. Although we used young mice (6–10 wk of age) maintained in specific pathogen-free facilities, we reasoned that there could potentially be a small number of CD4\(^{+}\) memory T cells present that would be capable of producing sufficient amounts of IL-4 to influence the predominant production of IL-4 in our cultures. Consistent with this notion, inclusion of an anti-IL-4 mAb in our cultures during the activation and expansion phase markedly decreased the percentage of cells producing IL-4 upon subsequent restimulation, but importantly had no effect on the expression of IFN-γ (data not shown). Because so many of our activated T cells produced IL-4...
FIGURE 4. Ectopic expression of caNFATc1 selectively enhances the expression of IFN-γ in CD4+ T cells maintained under neutral conditions. Purified CD4+ T cells from C57BL/6 (A), BALB/c (B), or DO11.10 × Rag1−/− (C) mice transduced with the indicated retrovirus were collected on day 7 and restimulated for 6 h with either medium alone (NS) or medium containing PMA plus ionomycin (P + I) and analyzed for expression of IFN-γ and IL-4 by intracellular cytokine staining. Data are presented as two-color dot plots showing IFN-γ (vertical axis) vs IL-4 (horizontal axis) after electronically gating on virally transduced populations. The percentage of cells in each quadrant is indicated. Data are representative of at least three independent experiments.

caNFA Tc1 enhances the expression of IFN-γ in developing Th1 cells

The effects of caNFATc1 on IFN-γ expression in CD4+ T cells maintained under neutral conditions prompted us to test the effects of caNFATc1 on cytokine production in cells specifically skewed toward either the Th1 or Th2 phenotype. To accomplish this, CD4+ T cells activated in the presence of either Th1- or Th2-polarizing conditions were transduced with either the control or caNFATc1 retrovirus and then maintained under the appropriate skewing conditions. As expected, when control virus-transduced cells maintained under Th1-skewing conditions were restimulated, a large percentage of the cells were found to produce IFN-γ (47%), with <1% of cells exhibiting production of IL-4, clearly indicating the efficiency of our Th1-skewing conditions (Fig. 5A). Consistent with our previous results, analysis of cells transduced with the caNFATc1 virus revealed a pronounced increase in the percentage of IFN-γ-expressing cells (73%) compared with control cells (47%). In fact, not only did we observe an increase in the total percentage of IFN-γ-expressing cells, but we also observed a noticeable increase in the level of IFN-γ expression produced per cell, as measured by an increase in the mean fluorescence intensity (514 vs 324). Importantly, expression of caNFATc1 had no effect on IL-4 production in these cells.

FIGURE 5. Effect of ectopic expression of caNFATc1 on IFN-γ and IL-4 expression in developing Th1 and Th2 cells. Activated CD4+ T cells from C57BL/6 mice were transduced with the indicated retrovirus in the presence of either Th1-skewing conditions (A) (10 ng/ml IL-2, 5 ng/ml IL-12, and 10 μg/ml anti-IL-4 mAb) or Th2-skewing conditions (B) (10 ng/ml IL-2, 50 ng/ml IL-4, 5 μg/ml anti-IL-12 mAb, and 5 μg/ml anti-IFN-γ mAb). Cells were expanded in the presence of the appropriate skewing conditions and on day 7 were washed, restimulated with either medium alone (NS) or medium containing PMA plus ionomycin (P + I), and then analyzed for expression of IFN-γ and IL-4 by intracellular cytokine staining. Data are presented as in Fig. 4. In the case of Th2-skewed cells (B), expression of IFN-γ vs IL-4 in the nontransduced cell populations from the same cultures is also shown. C, CD4+ T cells from C57BL/6 mice activated in the presence of Th2-skewing conditions were transduced with either control-H-2Kb or caNFATC1-H-2Kb retrovirus and on day 7 retrovirally infected cells were purified using paramagnetic anti-H-2Kb beads, washed, and restimulated (1.5 × 10⁶ cells/ml) with medium containing PMA plus ionomycin. After 24 h of stimulation, IFN-γ and IL-4 levels in the culture supernatants were assayed by ELISA as described in Materials and Methods. Data are given as mean values ± SD. Results are representative of three independent experiments.
Ectopic expression of caNFATc1 decreases production of IL-4 and promotes expression of IFN-γ in developing Th2 cells

Next, we examined the effects of caNFATc1 on cytokine expression in cells maintained under Th2-skewing conditions. As shown in Fig. 5B, stimulation of control virus-transduced cells expanded under Th2-polarizing conditions resulted in a population of cells exclusively producing IL-4, clearly demonstrating the efficiency of our Th2-skewing conditions. However, in marked contrast, CD4+ T cells that were transduced with the caNFATc1 virus under Th2-skewing conditions did not exhibit the expected Th2-like phenotype. Rather, we observed that following restimulation 25% of the cells expressed IFN-γ, with 15% of these cells producing solely IFN-γ and 10% producing both IFN-γ and IL-4. Remarkably, in addition to the enhancing effects of caNFATc1 on IFN-γ expression, we also observed an inhibitory effect of caNFATc1 on IL-4 expression, as the percentage of IL-4 producers in cells transduced with the caNFATc1 virus (34%) was decreased compared with the percentage of IL-4 producers observed in control cells (59%). These differential effects of caNFATc1 on the expression of IL-4 and IFN-γ in developing Th2 cells were confirmed by ELISA (Fig. 5C). Importantly, the effects of NFATc1 appear to be cell autonomous, because simultaneous analysis of the nontransduced cell population from the same culture indicate that they exclusively produce IL-4 at levels comparable to control cells (Fig. 5B). This latter result is especially important, because it indicates that the cultures were efficiently stimulated and that the diminished IL-4 production in the caNFATc1-transduced cells is a direct result of the intrinsic activity of caNFATc1.

Expression of caNFATc1 enhances the expression of functional PSGL-1 and induces high level expression of CD45RB

The selective effects of caNFATc1 on the expression of IFN-γ and the down-regulation of IL-4 production in Th2 cells suggested that expression of caNFATc1 might favor a Th1-like pattern of gene expression. To investigate this issue further we next analyzed the effects of caNFATc1 on the expression of cell surface markers that have been associated with the Th1 phenotype. While it has been difficult to identify cell surface molecules that absolutely define either Th1 or Th2 cells, a functional form of PSGL-1 has been shown to be preferentially expressed on activated Th1 cells (30). Using a P-selectin recombinant Ig fusion protein as a probe, we were able to assay cells for their expression of functional PSGL-1. As shown in Fig. 6A, cells transduced with the caNFATc1 virus exhibited significantly higher levels of expression of functional PSGL-1 following restimulation when compared with control virus-transduced cells.

In addition to PSGL-1, we also analyzed cells for their expression of CD45RB, as the level of expression of this CD45 isoform has been shown to subdivide functionally distinct CD4+ T cell populations. In this regard, CD4+ T cells expressing high levels of CD45RB have been shown to exhibit a number of properties commonly associated with Th1 effector cells. Thus, CD45RBhigh cells have been reported to produce high levels of IL-2 and IFN-γ but little IL-4, have been implicated as effectors in a proinflammatory autoimmune disease, and have been shown to protect BALB/c mice from infection with Leishmania major (43–45). As shown in Fig. 6B, CD4+ T cells transduced with the caNFATc1 virus expressed significantly higher levels of the CD45RB marker compared with cells transduced with the control virus (~4-fold). Hence, sustained NFAT signaling mediated by ectopic expression of caNFATc1 acts to enhance the expression of cell surface markers that appear to be preferentially associated with cells exhibiting a Th1 phenotype.

Expression of caNFATc1 leads to increased expression of FasL and enhanced AICD

Peripheral T cells are known to undergo AICD in response to repeated antigenic stimulation by a signaling pathway involving Fas and FasL (46). Moreover, increased expression of FasL and enhanced susceptibility to AICD are cellular properties that have previously been shown to be preferentially associated with Th1 effector cells (31–33). Because the NFAT family of transcription factors has been implicated in the T cell activation-inducible expression of FasL (47), we decided to investigate the effects of caNFATc1 on the expression of FasL and on AICD. As shown in Fig. 7A, T cells transduced with the caNFATc1 virus exhibited higher levels of FasL expression under either resting or stimulated conditions when compared with cells transduced with the control virus. This enhancing effect of caNFATc1 on FasL expression is consistent with the presence of functional NFAT binding sites within the FasL promoter (47), suggesting a direct transcriptional effect of caNFATc1 on FasL expression.

FIGURE 6. Effect of ectopic expression of caNFATc1 on expression of functional PSGL-1 and CD45RB in CD4+ T cells. Activated CD4+ T cells from C57BL/6 mice were transduced with the indicated retrovirus and expanded in medium containing IL-2. On day 7, cells were washed, restimulated with either medium alone (NS) or medium containing PMA plus ionomycin (P + I) for 8 h, and then analyzed for expression of functional PSGL-1 by staining with a P-selectin-Ig fusion protein (A) and CD45RB by flow cytometry (B). Data are presented as single-color histograms after electronically gating on virally transduced populations. The dotted lines represent staining with an isotype control reagent and specific staining is indicated by shading. The percentage of cells staining positive for each cell surface marker is indicated. Data are representative of at least two independent experiments.

FIGURE 7. Ectopic expression of caNFATc1 increases expression of FasL and enhances AICD

Peripheral T cells are known to undergo AICD in response to repeated antigenic stimulation by a signaling pathway involving Fas and FasL (46). Moreover, increased expression of FasL and enhanced susceptibility to AICD are cellular properties that have previously been shown to be preferentially associated with Th1 effector cells (31–33). Because the NFAT family of transcription factors has been implicated in the T cell activation-inducible expression of FasL (47), we decided to investigate the effects of caNFATc1 on the expression of FasL and on AICD. As shown in Fig. 7A, T cells transduced with the caNFATc1 virus exhibited higher levels of FasL expression under either resting or stimulated conditions when compared with cells transduced with the control virus. This enhancing effect of caNFATc1 on FasL expression is consistent with the presence of functional NFAT binding sites within the FasL promoter (47), suggesting a direct transcriptional effect of caNFATc1 on FasL expression.
EFFECTS OF caNFATc1 IN MURINE T CELLS

We next evaluated the effects of caNFATc1 expression on the induction of AICD. For these experiments, we took advantage of our H-2K^k retroviral constructs, as they allowed us to readily purify virally infected cells using anti-H-2K^k paramagnetic beads. It is important to note that these viruses gave essentially identical results to the previously described GFP viruses (data not shown). T cells transduced with either the control-H-2K^k or caNFATc1-H-2K^k retrovirus were purified using paramagnetic anti-H-2K^k beads and stimulated with medium containing PMA plus ionomycin. TUNEL assays were performed after 24 and 48 h of stimulation. The percentage of TUNEL-positive cells at each time point is indicated by shading. The percentage of cells staining positive for FasL is indicated by specific gating on virally transduced populations. The dotted lines represent data are presented as single-color histograms after electronically gating on virally transduced populations. The dotted lines represent staining with the appropriate isotype control Ab and specific staining is indicated by shading. The percentage of cells staining positive for FasL is indicated. B, Activated CD4^+ T cells from C57BL/6 mice transduced with either control-H-2K^k or caNFATc1-H-2K^k retrovirus were purified using paramagnetic anti-H-2K^k beads and stimulated with medium containing PMA plus ionomycin. TUNEL assays were performed after 24 and 48 h of stimulation. The percentage of TUNEL-positive cells at each time point is indicated. Control-H-2K^k-transduced cells are represented by the filled bars and the caNFATc1-H-2K^k-transduced cells are represented by the shaded bars. Data are representative of at least two independent experiments.

FIGURE 7. Ectopic expression of caNFATc1 in CD4^+ T cells enhances expression of FasL and increases susceptibility to AICD. A, CD4^+ T cells from C57BL/6 mice transduced with the indicated retrovirus were restimulated with either medium alone (NS) or medium containing PMA plus ionomycin (P + I) for 8 h and then analyzed for expression of FasL by flow cytometry. Data are presented as single-color histograms after electronically gating on virally transduced populations. The dotted lines represent staining with the appropriate isotype control Ab and specific staining is indicated by shading. The percentage of cells staining positive for FasL is indicated by shading. The percentage of cells staining positive for FasL is indicated. B, Activated CD4^+ T cells from C57BL/6 mice transduced with either control-H-2K^k or caNFATc1-H-2K^k retrovirus were purified using paramagnetic anti-H-2K^k beads and stimulated with medium containing PMA plus ionomycin. TUNEL assays were performed after 24 and 48 h of stimulation. The percentage of TUNEL-positive cells at each time point is indicated. Control-H-2K^k-transduced cells are represented by the filled bars and the caNFATc1-H-2K^k-transduced cells are represented by the shaded bars. Data are representative of at least two independent experiments.

Discussion

The NFAT family of transcription factors has been strongly implicated in the T cell activation-dependent regulation of numerous cytokine genes and is therefore believed to play a pivotal role in the TCR-induced expression of cytokine genes during Th cell differentiation (3–5). While the NFAT signaling pathway is known to be exquisitely sensitive to changes in the [Ca^{2+}]_i (6, 7, 12), which is believed to play an important role in coupling differences in TCR-induced signaling events into changes in gene expression, the role of spatiotemporally distinct patterns of NFAT signaling in the regulation of specific cytokine genes is not well understood. In the current study, we have used an efficient retroviral delivery system to introduce a calcineurin-independent, constitutively active mutant version of NFATc1 (caNFATc1) into primary murine CD4^+ T cells, thereby allowing us to examine the functional consequences of sustained NFAT activity on gene expression in primary T cells.

While we found that ectopic expression of caNFATc1 clearly affected the expression of the T cell activation markers CD25, CD40L, and CD62L, and was able to synergize with PMA to induce expression of IL-2, our most striking finding was the selective enhancing effect of caNFATc1 on the expression of the prototypical Th1 cytokine, IFN-γ. In this regard, we found that when CD4^+ T cells were grown under neutral conditions that did not favor either Th1 or Th2 development, ectopic expression of caNFATc1 resulted in a substantial selective increase in the percentage of cells producing IFN-γ. Interestingly, under these conditions, we observed little, if any, effect of caNFATc1 on IL-4 production. This was surprising because NFAT has been shown to regulate the activity of the IL-4 promoter in transiently transfected cells, and T cells from NFATc1-deficient mice have been reported to produce diminished amounts of IL-4 in response to anti-CD3 mAb stimulation (22, 23, 48). However, while we did not see an effect of caNFATc1 on the percentage of IL-4-producing cells per se, we did observe an increase in the percentage of cells producing both IFN-γ and IL-4, together with a concomitant reduction in cells producing only IL-4, suggesting that the expression of caNFATc1 is sufficient to induce IFN-γ expression in cells producing IL-4. These double cytokine-producing cells are suggestive of Th0 cells, which have been reported to be preferentially generated by repetitive rounds of antigenic stimulation (49). Hence, it is possible that expression of caNFATc1 mimics repeated T cell stimulation, thereby favoring the generation of Th0 cells under neutral nonpolarizing cytokine conditions.

Because we had found that ectopic expression of caNFATc1 was able to selectively increase the percentage of IFN-γ-producing cells under neutral cytokine conditions, we were naturally interested in examining the effects of caNFATc1 on IFN-γ and IL-4 expression in cells induced to differentiate toward either the Th1 or Th2 phenotype. As might have been expected from our previous data, cells expressing caNFATc1 that were maintained under strong Th1-inducing conditions exhibited increased numbers of cells producing IFN-γ compared with controls (Fig. 5A). This effect of caNFATc1 on the expression of IFN-γ is consistent with the presence of NFAT binding sites within the cis-acting regulatory elements of the IFN-γ gene and the ability of NFATc1 to transactivate an IFN-γ promoter reporter construct in a transient...
transfection assay (50, 51). However, despite the presence of multiple functional NFAT binding sites in the IL-4 promoter (41, 42), ectopic expression of caNFATc1 in developing Th2 cells did not enhance the production of IL-4. Quite to the contrary, expression of caNFATc1 in cells skewed toward the Th2 phenotype actually diminished the percentage of cells expressing IL-4 and unexpectedly promoted the expression of IFN-γ (Fig. 5B). These effects of caNFATc1 in developing Th2 cells are all the more remarkable because it is well established that under normal Th2-skewing conditions IL-4 acts to strongly up-regulate expression of the endogenous IL-4 gene and potently suppress the expression of IFN-γ (1).

In addition to its effects on cytokine gene expression, we also found that ectopic expression of caNFATc1 enhanced the expression of a number of cell surface molecules and cellular traits that appear to be preferentially associated with Th1 effector cells, including enhanced expression of functional PSGL-1 (30), high-level expression of CD45RB (43–45), increased Fasl expression, and enhanced susceptibility to AICD (31–33). Taken together, these data suggest that ectopic expression of caNFATc1 appears to preferentially favor a Th1-like pattern of gene expression.

Interestingly, our findings regarding the effects of caNFATc1 on the expression of IFN-γ and IL-4 appear to conflict with the previous analysis of mice deficient in NFATc1. In this regard, NFATc1-deficient splenocytes or lymph node cells have been reported to produce diminished levels of IL-4 in response to mitogenic stimulation, which has been taken as evidence that NFATc1 is specifically involved in the regulation of Th2 cytokines (22, 23). However, our finding that ectopic expression of caNFATc1 acts to enhance expression of the prototypic Th1 cytokine IFN-γ, yet inhibit expression of the Th2 cytokine IL-4, suggests that NFATc1 per se is not likely to be uniquely associated with the regulation of Th2 cytokines. Indeed, recent analysis of NFATc1/NFATc2 doubly deficient T cells (29) demonstrating a profound defect in cytokine production relative to T cells deficient in either NFATc1 or NFATc2 alone (22–25) suggests that there is in fact likely to be a considerable degree of overlap between the specific cytokine target genes of both NFATc1 and NFATc2. This notion is further supported by experiments indicating that both NFATc1 and NFATc2 exhibit very similar in vitro DNA binding specificities (4, 16, 17). Accordingly, it is important to note that the effects on gene expression that we observe with caNFATc1 may not necessarily be specific to this NFAT family member per se, but rather may merely reflect a general property of the sustained activity of the NFAT signaling pathway caused by the ectopic expression of a constitutively active NFAT mutant.

The Th1/Th2 cell fate decision is regulated by a number of different factors, including the cytokine environment, level of co-stimulation, and the degree of TCR stimulation (1, 52). In this latter case, high doses of Ag or antigenic peptides that exhibit high affinity for the TCR appear to favor the development of Th1 cells, whereas low doses of Ag or Ag with low affinity for the TCR have been shown to preferentially induce Th2 differentiation (52–54). These different types of antigenic stimulation are known to induce different degrees of TCR signaling that translate into the generation of distinct patterns of intracellular biochemical second messengers. Specifically, Ags with different affinities for the TCR are known to elicit distinct changes in [Ca^{2+}], (54, 55); high-affinity Ags induce sustained/high-frequency calcium oscillations, whereas low-affinity Ags induce transient/low-frequency calcium oscillations. In fact, recent studies have demonstrated that the quantitative nature of the calcium signal can indeed influence the Th1/Th2 cell fate decision, with a strong increase in [Ca^{2+}], appearing to preferentially favor the development of Th1 cells (54, 56).

Given the exquisite calcium sensitivity of the NFAT family of transcription factors and their ability to respond to quantitatively distinct changes in [Ca^{2+}], in a highly stimulus-specific fashion (12), NFAT proteins are perfectly poised to translate quantitatively distinct Ag-induced changes in [Ca^{2+}], into specific transcriptional responses capable of influencing the outcome of Th cell differentiation. In fact, evidence to support a role for quantitative differences in the calcineurin/NFAT signaling pathway regulating the Th1/Th2 cell fate decision has been provided by the observation that partial inhibition of calcineurin, the direct upstream activator of NFAT proteins, can redirect Th1-skewed cells toward the Th2 phenotype (56). In this case, reduced signaling through the calcineurin/NFAT signaling pathway appears to favor a Th2 pattern of differentiation. Conversely, our current data demonstrate that increased and sustained signaling through the NFAT signaling pathway caused by ectopic expression of caNFATc1 appears to favor a Th1-like pattern of gene expression. In this instance we believe that, as a result of its constitutively active state, ectopic expression of caNFATc1 is able to phenocopy the effects of sustained signaling through the calcium/calcineurin signaling pathway, and just like a strong calcium signal (54, 56), is able to induce a transcriptional response that preferentially favors a Th1-like pattern of gene expression. Taken together with recent results demonstrating that the extent and duration of NFAT activation can significantly affect the qualitative pattern of gene transcription induced during T cell activation (13), these two complementary sets of data suggest that the degree of signaling through the calcineurin/NFAT signaling pathway is likely to play an important role in determining the differential pattern of cytokine gene expression induced during Th cell differentiation.

What are likely to be the underlying molecular mechanisms that account for the effects of caNFATc1 on gene expression? While the differential expression of IFN-γ and IL-4 is known to be influenced by a variety of cytokines (1), it is clear from our analysis of caNFATc1-transduced and nontransduced cell populations that the effects of caNFATc1 are cell autonomous and are therefore unlikely to involve the production of a secreted paracrine factor. Hence, caNFATc1 is likely to act in a cell intrinsic fashion to directly influence cytokine gene expression. Because the cis-acting regulatory elements of both IFN-γ and IL-4 are known to contain functional NFAT binding sites (41, 42, 50, 51), the most straightforward model to explain the differential effects of caNFATc1 on the expression of IFN-γ and IL-4 involves the direct action of caNFATc1 on the regulatory elements of these genes. While a direct transcriptional effect of caNFATc1 could easily account for the greatly enhanced expression of IFN-γ, it does not readily explain the inhibitory effect of caNFATc1 on IL-4 expression observed in developing Th2 cells.

One possibility is that while caNFATc1 might act as a positively acting transcription factor at the IFN-γ locus by potentially promoting chromatin remodeling of the locus and directly increasing IFN-γ transcription, it may instead act as a context-specific transcriptional repressor on the IL-4 promoter in developing Th2 cells. Although no such repressive role for NFATc1 has been described, it is interesting to note that another NFAT family member, NFATc2, is believed to exhibit inhibitory activity and can apparently inhibit the expression of IL-4 in vivo (26). Whether, like NFATc2, NFATc1 can in some cases either down-regulate or directly repress transcription of certain genes remains to be determined. An alternative explanation for the inhibitory effect of caNFATc1 on IL-4 production in developing Th2 cells is that ectopic expression of caNFATc1 might interfere with the IL-4-dependent signaling pathway responsible for inducing IL-4 gene expression during Th2 differentiation. In this regard, IL-4 expression is known to require the actions of both the
GATA-3 and c-maf transcription factors (57, 58), while in addition GATA-3 has been shown to play a role in repressing the expression of IFN-γ in developing Th2 cells (59). Because NFAT proteins have been reported to interact with c-maf (60) and NFATc1 is known to specifically interact with another GATA factor, GATA-2 in skeletal muscle (61), it is possible that caNFATc1 acts by directly antagonizing the activities of c-maf and GATA-3, thereby blocking the induction of IL-4 expression and preventing the repression of IFN-γ expression in developing Th2 cells. Finally, it is possible that ectopic expression of caNFATc1 mediates its effects via an indirect mechanism by inducing the expression of a target gene(s) that in turn is either capable of differentially affecting the expression of IFN-γ and IL-4, or is able to directly influence the Th1/Th2 cell fate decision. In this case, a potential candidate gene is T-bet, a recently described TCR-induced master regulatory transcription factor gene implicated in the induction of Th1 cell differentiation (62). Ectopic expression of T-bet has been shown to selectively increase the number of cells expressing IFN-γ by inducing chromatin remodeling of the IFN-γ locus (62, 63). Moreover, expression of T-bet in Th2 cells has been reported to redirect them toward the Th1 phenotype by inhibiting production of IL-4, thus altering the balance of pro-Th1 cytokines vs. anti-Th1 cytokines. This finding is consistent with recent results demonstrating the profound effect of the degree of NFAT signaling on the pattern of transcriptional events induced during T cell activation (13), and serve to underscore the potentially important relationship between the quantitative extent of NFAT signaling and the qualitative pattern of gene expression induced during Th cell differentiation.

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References


